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14. ABSTRACT

The goal of this proposal is to develop a novel bio-activated, aligned, nanofibrous scaffold that will serve as mechanical and biological support for the repair of radial tears of the meniscus. The hypothesis is that scaffolds consisting of aligned polymeric fibers, which structurally and mechanically mimic tendon and fibrocartilage, may be applied as a patch in alignment with the fibers of the tissue to be repaired, i.e., the meniscus with radial tear, to strengthen mechanically the surgical meniscal repair, and to subsequently guide tissue regeneration, for example, by seeded tissue progenitor cells.

To achieve this objective, the first step is to develop an aligned nanofibrous scaffold (NFS) that meets the mechanical requirements of the native meniscal matrix and provides suture retention. This will be done by combining nanofibers composed of FDA-approved biodegradable polymers to produce a biocompatible scaffold, which will provide mechanical support to the healing meniscus. To support suture retention, a second layer of non-aligned fibers will be coated onto the aligned fibers. Secondly, the NFS will be bio-enhanced by impregnation with an extract derived from decellularized meniscus matrix, which contains molecules and growth factors specific to this tissue, to increase the formation of fibrocartilage by adult stem cells seeded within the scaffold. This bio-activation should enhance the biological integration, i.e. adhesion, and tissue regenerating activity of the NFS in meniscus repair. Finally, we will test the ability of the bio-activated, aligned NFS sheath to enhance meniscus repair when combined with stem cell-based wound bonding strategies and standard suture repair using an in vitro model of meniscal repair (employed to elucidate the optimal combination of materials developed here) and in vivo, repairing surgically-induced radial defects in a goat.

15. SUBJECT TERMS

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The meniscus is the most commonly injured structure of the knee, disproportionately affecting active populations such as military personnel. At present, removal of the torn tissues (i.e., partial meniscectomy) is the standard of care but predisposes the patient to rapid joint degeneration (i.e., osteoarthritis). Tissue engineering approaches, including the combination of cells, scaffolds, and bioactive agents (e.g., growth factors), have been explored as a means of bolstering the poor intrinsic healing capacity of the meniscus. Aligned electrospun nano/microfibers comprising engineered scaffolds can mimic the ultrastructure of the native meniscal extracellular matrix (ECM); when seeded with adult mesenchymal stem cells (MSCs), the nanofibers direct MSC orientation with corresponding upregulation of fibrochondrogenic differentiation. Similarly, photocrosslinkable hydrogels derived from natural ECM proteins (e.g., collagen, gelatin) can deliver MSCs under point-of-care procedures to a tear site and promote subsequent neotissue formation. Supplementation of these hydrogels with bioactive agents, such as growth factors or soluble ECM fractions, can enhance tissue-specific neotissue formation. **The purpose of this project is to combine a biomimetic scaffold of electrospun nanofibers with a meniscal ECM-enhanced, MSC-seeded photocrosslinkable hydrogel to enhance healing of a radial meniscus tear, as evaluated in both an *in vitro* explant and *in vivo* goat model.**

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).
Meniscus tissue engineering, electrospun scaffold, hydrogel, extracellular matrix, mesenchymal stem cell

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the USAMRAA Grants Officer whenever there are significant changes in the project or its direction.

- What were the major goals and objectives of the project?
- What was accomplished under these goals?
- What opportunities for training and professional development did the project provide?
- How were the results disseminated to communities of interest?
- What do you plan to do during the next reporting period to accomplish the goals and objectives?

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Generally, the goals will not change from one reporting period to the next and are unlikely to change during the final reporting period. However, if the awarding agency approved changes to the goals during the reporting period, list the revised goals and objectives. Also explain any significant changes in approach or methods from the agency approved application or plan.

The project SOW contains three aims:

1. Develop aligned nanofibrous scaffold (NFS) to meet the mechanical requirements of the native meniscal matrix and provide suture retention
2. Develop and assess adult stem cell-seeded bioactivated NFS as a potential meniscal repair component
3. Verify the ability of the developed aligned NFS to promote meniscal repair in vitro and in a large animal model in vivo; the aligned NFS is to be combined with an ECM-enhanced photocrosslinkable hydrogel that will be injected into the tear site.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Results and discussion from the previous annual report (2015-2016) have been retained below for continuity. Past results are indicated in section headings in **RED**, while any new results/discussion are indicated by **GREEN**. With the exception of addressing two questions provided by the reviewer in response to our previous report, **there have been no changes to Aims 1 and 2**. The original project proposed to develop an aligned nanofibrous scaffold, that when combined with an MSC-seeded photocrosslinkable hydrogel, could enhance healing of a radial meniscus tear. Greater emphasis was originally given to the nanofibrous scaffold as compared to the MSC-seeded hydrogel.

As highlighted in the previous annual report, and further detailed in the resulting publication (**Rothrauff et al. *J. Exp. Orthop.* 2016; 3(1): 23**), we succeeded in creating an electrospun scaffold with material properties approaching those of native menisci. However, the suture retention strength of the scaffolds was insufficient to withstand shear forces expected in surgical repairs. Additional pilot studies over the past year identified difficulties in seamlessly incorporating the scaffold sheet into a surgical repair. At the same time, parallel studies to optimize the MSC-seeded hydrogel yielded exciting results in our established in vitro model of a radial meniscus tear. Consequently, and in consideration of the limited time to complete the project, we chose not to include the nanofibrous scaffold within Aim 3, but retained the optimized photocrosslinkable MSC-seeded. As detailed below, the results thus far have been promising, with final analyses expected to conclude by the end of the no-cost extension period (December 2017).

Accomplishments are organized within each aim:

1. **Aim 1: Develop aligned nanofibrous scaffold (NFS) to meet the mechanical requirements of the native meniscal matrix and provide suture retention (NO NEW RESULTS)**

Aim 1 Results (*PAST RESULTS, 2015 – 2016*):

Individual layers of poly-ε-caprolactone (PCL) nanofibers were electrospun in three orientations – aligned longitudinal, aligned transverse, and random (**Figure 1D-F**) – and combined as multilayered scaffolds of three patterns – aligned, random, and biomimetic (**Figure 1G-I**). The biomimetic scaffold contained, from deep to superficial, layers of aligned longitudinal, aligned transverse, aligned longitudinal, and random fibers, to mimic the circumferential, radial, and surface fiber ultrastructure of the native meniscus, respectively (**Figure 1**).

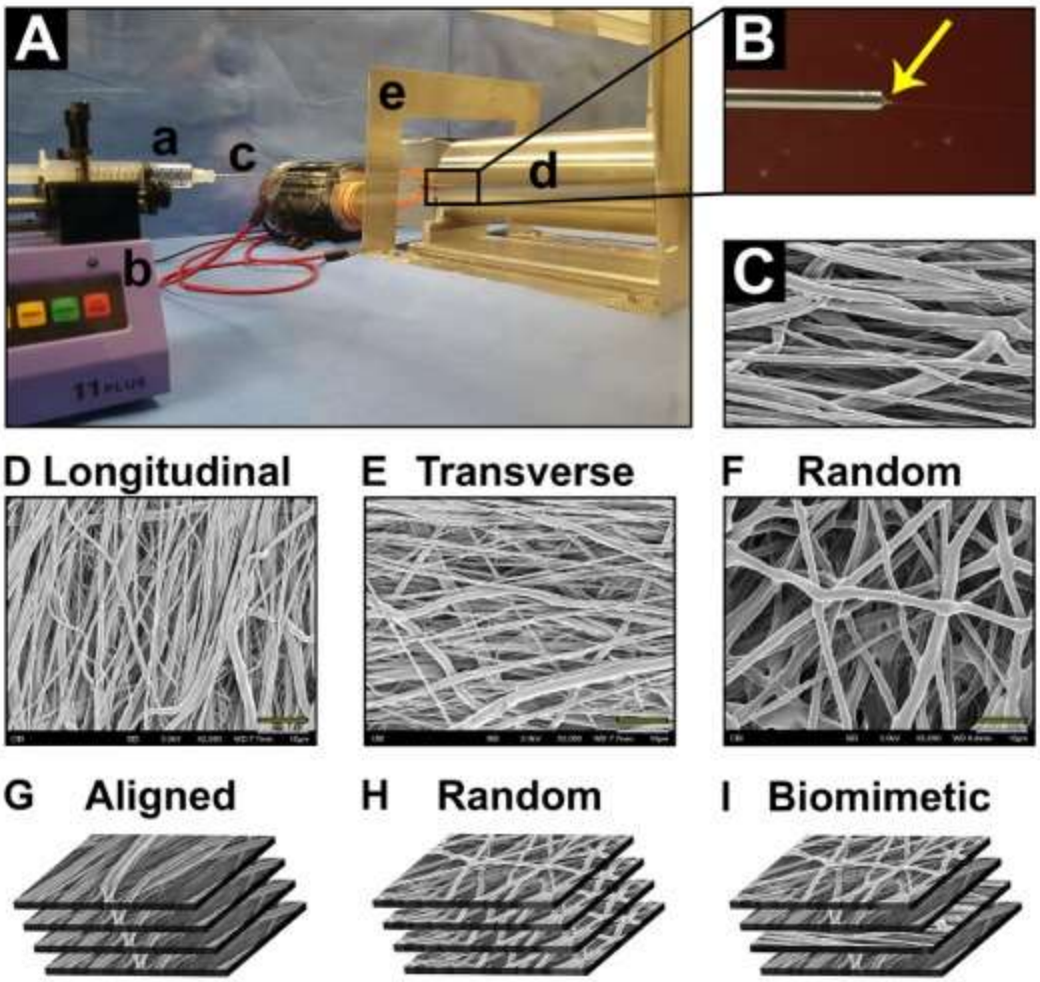


Figure 1. Fabrication of multilayered electrospun scaffolds. (A) Electrospinning apparatus consisting of (a) syringe with polymer solution, (b) syringe pump, (c) 18-gauge blunt tip needle, (d) rotating mandrel, and (e) aluminum shield. (B) Taylor cone (arrow) with emerging polymer fiber creates (C) nanofibrous sheet. (D-F) SEM images of fiber orientations comprising individual layers. Scale bar, 10 μm. (G-I) Individual layers are combined to form three types of multilayered scaffolds, (G) aligned, (H) random, and (I) biomimetic (consisting of alternating layers of aligned and random layers).

The three multilayered scaffolds were loaded under uniaxial tension in both the parallel and perpendicular direction, and both structural and material properties were determined. The aligned scaffolds showed the greatest degree of anisotropy, with the highest modulus of all conditions when tension was applied in the direction of the PCL fibers (i.e., parallel). However, the biomimetic scaffold was not significantly weaker in the parallel direction than aligned scaffolds, but superior to other designs in the perpendicular direction (**Figure 2A**). Additionally, suture retention strength was highest in the biomimetic scaffold (**Figure 2B**)

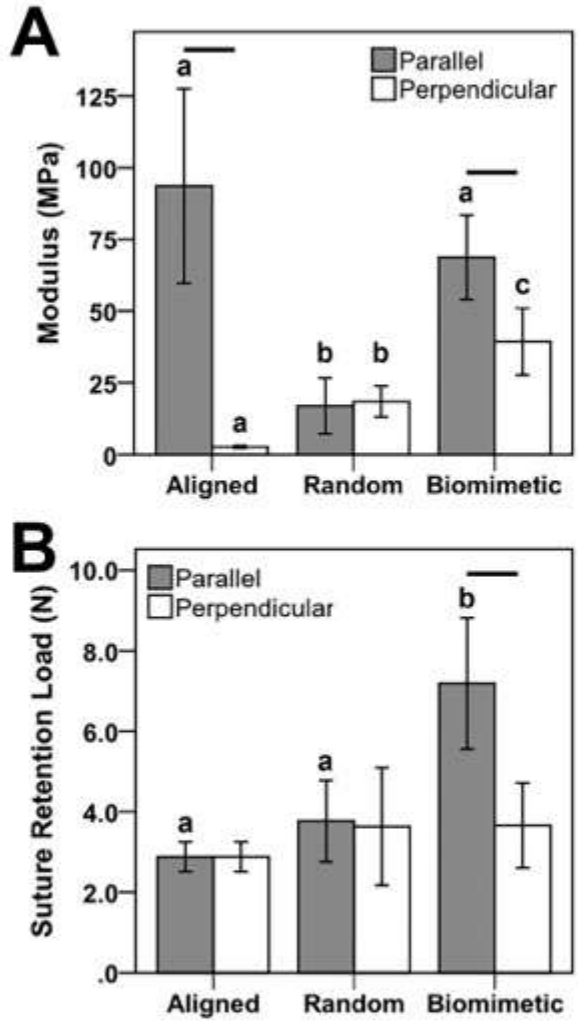
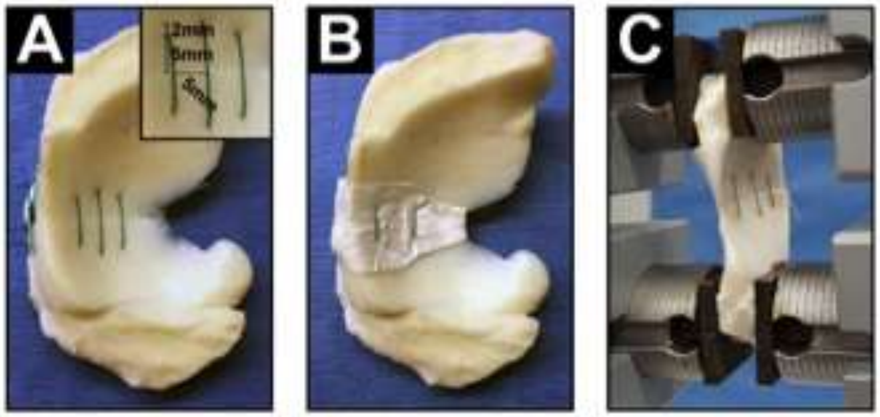


Figure 2. Moduli and suture retention strength of multilayered scaffolds. (A) Tensile modulus of three scaffold designs in parallel (i.e., circumferential) and perpendicular (i.e., radial) direction. (B) Ultimate suture retention load by scaffold design. * (p <0.05) and # (p <0.001) indicate significant difference across scaffold types for a given direction. Horizontal lines above columns indicate a significant difference (p < 0.001) between directions for a given scaffold type.

Given the superiority of the biomimetic scaffold design in terms of material properties and suture retention strength, it was incorporated as a sheath within a horizontal mattress suture repair of a radial tear simulated on a bovine meniscus explant (**Figure 3A,B**). The meniscus explanted was then cyclically loaded for 500 cycles (5N-20N) before loading to failure (**Figure 3C**). Testing revealed that the scaffold did not compromise, but did not improve, the residual gap formation of structural properties of the suture repair group (**Tables 1,2**).



Cycle	Native	Suture Repair	Scaffold-Augmented
1	0.26 ± 0.16[a]	1.14 ± 0.28	1.27 ± 0.38
10	0.40 ± 0.23[a]	1.75 ± 0.40	1.99 ± 0.33
50	0.55 ± 0.33[a]	2.57 ± 0.57	2.93 ± 0.35
100	0.66 ± 0.39[a]	3.15 ± 0.75	3.58 ± 0.47
250	0.86 ± 0.51[a]	4.29 ± 1.17	4.88 ± 0.80
500	0.93 ± 0.49[a]	4.78 ± 1.24	5.05 ± 0.89

	Native	Suture Repair	Scaffold-Augmented
Ultimate Load (N)[a]	437.3 ± 117.5	124.4 ± 21.4	137.1 ± 31.0
Ultimate Elongation (mm)[b]	5.12 ± 1.55	10.14 ± 4.61	12.09 ± 5.89
Stiffness (N/mm)	141.0 ± 42.4[a]	18.4 ± 4.7	20.8 ± 3.6

Figure 3. Suture repair of meniscal tears and mechanical testing set-up. (A) Suture repair of fully transected meniscus. Inset shows dimensions of suture placement. (B) Scaffold-augmented repair. (C) Suture repaired meniscus clamped in materials testing machine prior to tensile loading protocol. **Table 1. Residual Elongation (mm) During 500 Cycles Between 5N to 20N.** [a] Native control significantly less ($p < 0.001$) than either repair group at given cycle. **Table 2. Mechanical Properties of Native and Repaired Menisci Pulled to Failure.** [a] Native control significantly greater ($p < 0.001$) than either repair group. [b] Scaffold-augmented group significantly greater ($p = 0.022$) than native control.

Aim 1 Discussion:

We successfully developed an aligned electrospun nanofibrous scaffold (i.e., biomimetic) that approaches the material properties of native meniscus. The scaffold was stably incorporated as part of a clinically relevant suture repair, but did not enhance cyclic or load properties of the repair. The ultimate loads and stiffness for repaired constructs (i.e., suture or scaffold-augmented groups) met or exceeded values reported in the literature. As all repairs failed by suture breakage, the limitation in enhancing the strength of suture repairs is the suture material itself, not the strength of the meniscus tissue nor the overlying scaffold. To that end, improving the material strength of the scaffold, or its suture retention strength, would NOT enhance the strength of the repair. Nevertheless, the inclusion of the scaffold could serve several purposes: (1) directing ECM deposition in the direction of electrospun fibers by endogenously recruited or exogenously seeded progenitor cells; (2) protecting an MSC-seeded hydrogel photocrosslinked within the tear site.

2. **<u>Aim 2</u>: Develop and assess adult stem cell-seeded bioactivated NFS as a potential meniscal repair component (<u>NO NEW RESULTS</u>)**

Aim 2 Results (*PAST RESULTS, 2015 – 2016*):

Aim 2 assesses the ability of the NFS to support cell attachment and matrix deposition. As stated in the original project SOW, the NFS is further enhanced with a urea-extracted fraction of ECM derived from the ***outer*** meniscus. The NFS will be combined with an MSC-seeded photocrosslinkable hydrogel that could be further enhanced with a urea-extracted fraction of the ECM derived from the ***inner*** meniscus. To obtain the urea-extracted fraction of meniscus ECM, we adapted a protocol previously established in our lab for tendon ECM.[1] As shown in **Figure 4**, menisci from 6-8 week old bovine hindlimbs were halved, minced, crymilled, and solubilized through one of two methods – (1) urea extraction or (2) pepsin digestion. The urea-extracted fractions were enriched for small to medium weight non-collagenous proteins, while pepsin digestion produced a homogenous slurry that contained mostly collagen (**Figure 4E-H**).

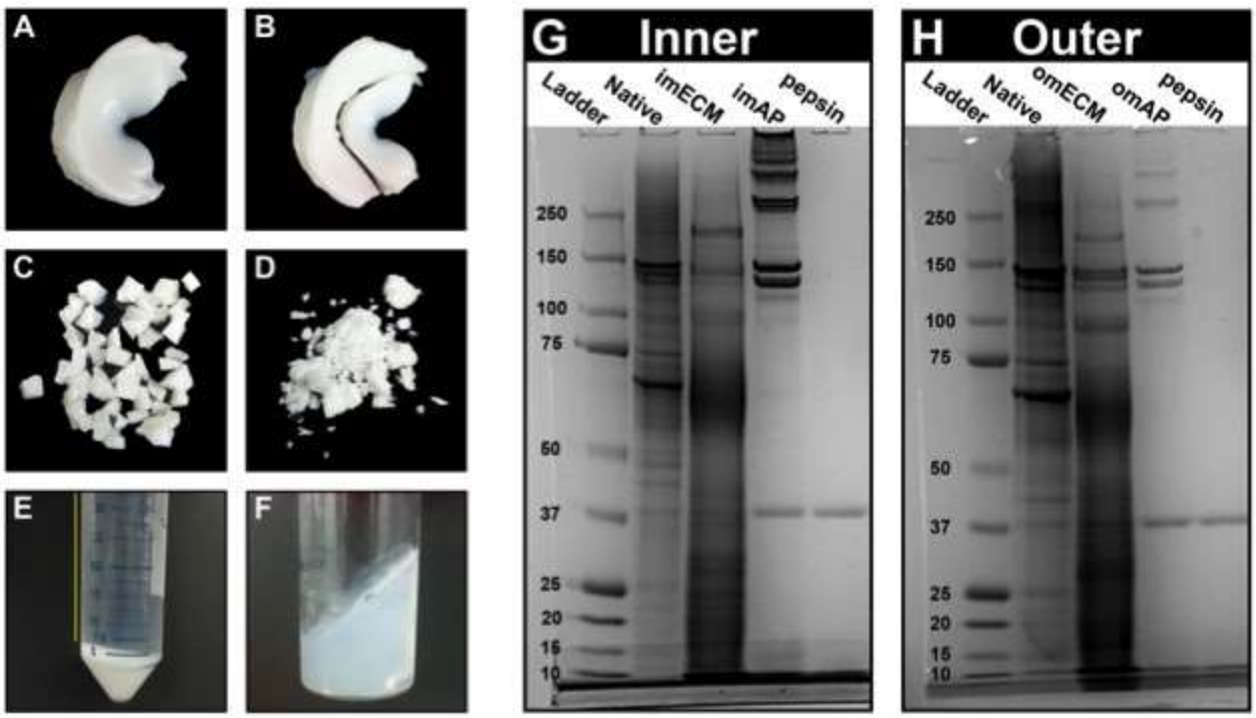


Figure 4. Solubilization of ECM from inner and outer meniscus. (A) Whole menisci were obtained from 6-8 week old cow hindlimbs, (B) halved, and (C) manually minced (8-27 mm^3). Following decellularization, (D) tissues were cryomilled and soluble fractions were obtained either by (E) urea-extraction (supernatant was retained, yellow line) or (F) acid-pepsin digestion. SDS-PAGE of (G) inner meniscus and (H) outer meniscus tissues and soluble preparations demonstrate that urea extraction retained low- and moderate-weight proteins while pepsin digestion yielded mostly collagen.

To determine the bioactivity of soluble meniscal ECMs, human bone marrow-derived MSCs were grown on 2D tissue culture plastic for up to 7 days. Basal medium (**control**) consisted of DMEM, 1% v/v PSF, 1% v/v ITS. Media were further supplemented with one of four soluble ECMs, all at a final concentration of 50 μg/mL – (1) urea-soluble imECM, (2) urea-soluble omECM, (3) pepsin-digested imAP, or (4) pepsin-digested omAP. Medium was changed every 2 days. Preliminary studies revealed that pepsin-digested ECM (imAP, omAP) did not promote region-specific differentiation of MSCs seeded on 2D plastic. Conversely, urea-extracted fractions of meniscus ECM supported increased cell proliferation (**Figure 5A-D**) and fibrochondrogenic differentiation. In particular, while both soluble ECMs derived from the inner meniscus (imECM) and outer meniscus (omECM) upregulated fibrochondrogenic markers Sox9, Collagen type II (Col2), and Collagen type I (Col1), these results were more strongly promoted by imECM (**Figure 5E**).

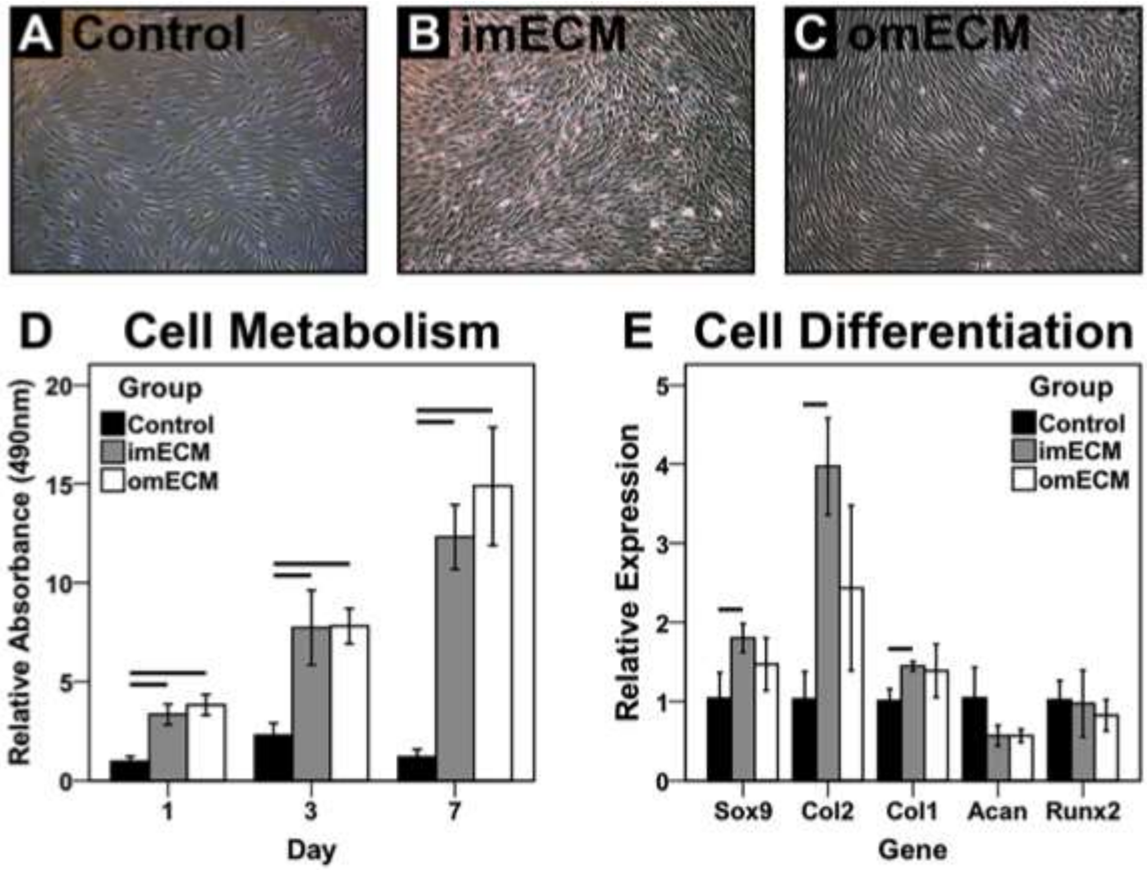


Figure 5. Bioactivity of soluble ECM extracts on MSCs in 2D culture. (A-C) phase contrast microscopy. (D) MTS assay measuring cell metabolism; n = 6-8 per condition; Lines indicate significant difference between groups on given day, $p < 0.05$. (E) Gene expression analysis on day 3; n=3 independent trials, each performed in biological triplicate; Lines indicate significant difference between groups, $p < 0.05$.

15.0×10^6 MSCs/mL (derived from human bone marrow) were suspended in 10% w/v methacrylated gelatin (GelMA) hydrogels containing 0.25% photoinitiator (LAP) and supplemented with one of the following – (1) 1X PBS (Control), (2) 500 μg/mL imECM, or (3) 500 μg/mL omECM. Hydrogels were crosslinked by exposure to visible light (450-490nm) for 2 minutes and cultured for up to 42 days in chondrogenic medium (supplemented with 10 ng/mL TGF-β3). As shown in **Figure 6**, both imECM and omECM enhanced Col2 and proteoglycan deposition, with imECM being superior to omECM.

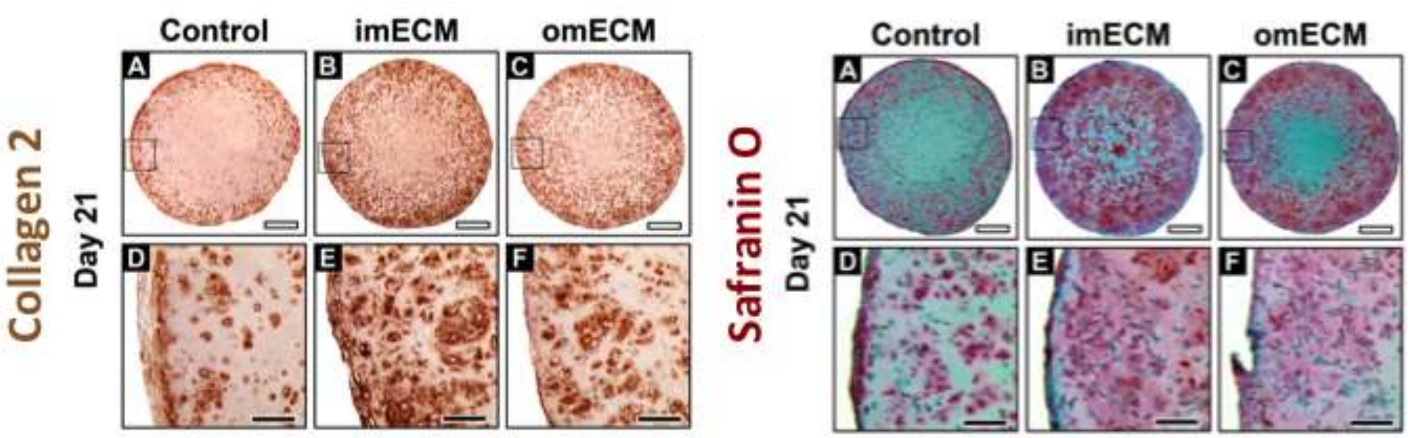


Figure 6. Immunohistochemical staining of Col2 (left) and histological staining (Safranin O/fast green; right) of ECM-enhanced GelMA hydrogels seeded with human bone marrow-derived MSCs. Dark Brown = Col2; Red = Proteoglycan.

To explore the bioactivity of NFSs, MSCs were seeded on electrospun sheets of random or aligned nanofibers (**Figure 7**). The fiber orientation influenced cell morphology, with MSCs seeded on random fibers demonstrating no obvious directionality while those cultured on aligned fibers adopted an elongated morphology parallel to the fiber axis. Preliminary studies in which the PCL fibers were soaked in omECM showed relatively rapid loss of the omECM coating when exposed to culture medium, suggesting that protein adsorption is an insufficient method for stably functionalizing NFSs.

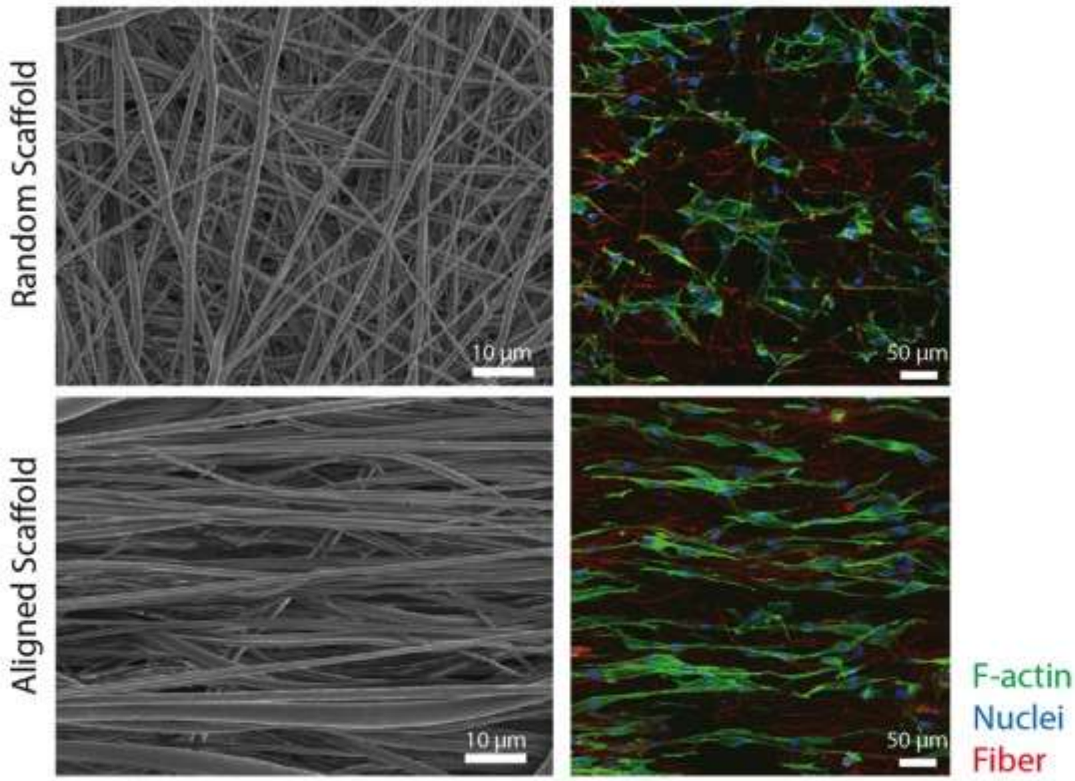


Figure 7. Morphology of MSCs cultured on scaffolds. MSCs seeded on random scaffold (upper left, SEM) exhibited polygonal shape without uniformity in orientation (upper right, confocal microscopy). In contrast, MSCs seeded on aligned scaffolds (lower left, SEM) adopted an elongated morphology and were orientated in the direction of the fibers (lower right, confocal microscopy; F-actin, green; nuclei, blue; microfiber, red).

Aim 2 Discussion:

The Biomimetic scaffold developed in Aim 1 was capable of supporting MSC attachment and orientation-mediated changes in cell morphology. While omECM and imECM enhanced fibrochondrogenic differentiation of MSCs cultured on 2D plastic or in photocrosslinkable GelMA hydrogels, this effect was stronger for imECM. Given the loss of omECM coating from NFSs when relying on protein adsorption, coupled with the weaker fibrochondrogenic effect of omECM (vs. imECM), we are pursuing Aim 3 (below) without further modification of the biomimetic scaffold. At the same time, imECM-enhanced GelMA hydrogels to promote robust chondrogenic differentiation of encapsulated MSCs. Furthermore, encapsulating TGF-β within the hydrogel produces a comparable effect as adding TGF-β as a medium supplement, permitting us to use GelMA hydrogels containing imECM, TGF-β, and MSCs, in point-of-care repairs of meniscus tears.

3. **Aim 3: Verify the ability of the developed aligned NFS to promote meniscus repair *in vitro* and in a large animal model *in vivo*. (ALL NEW RESULTS)**

Aim 3 Results:

In Vitro Repair:

Building on earlier work described in Aim 2 (**Figures 4-6**), we explored the independent effects of urea-extracted ECM derived from inner (imECM) and outer (omECM) menisci, as compared to TGF-β alone, in terms of promoting chondrogenic differentiation of MSCs seeded in a photocrosslinkable GelMA hydrogel. The soluble ECM (500 μg/mL) was added to the GelMA prior to photogelation, as described above. However, constructs were cultured in medium ***without*** TGF-β supplementation. Both imECM and omECM independently upregulated fibrochondrogenic differentiation (data not shown), as measured by upregulated expression of collagen type I, collagen type II, and aggrecan, with the former preferentially promoting a chondrogenic phenotype (i.e., higher Col2, acan) and the latter promoting a fibroblastic phenotype (i.e., higher Col1). However, the relative increases in gene expression for the aforementioned genes were only 2-5 fold. In comparison, for constructs cultured in medium supplemented with 10 ng/mL TGF-β3, expression of chondrogenic genes increased 10-10,000 fold. Robust deposition of chondrogenic proteins (i.e., proteoglycan, Collagen type II) was only seen in constructs cultured in medium containing TGF-β3. **These results suggested that TGF-β was necessary for robust neo-cartilage formation.**

On the other hand, the delivery of TGF-β for in vivo application likely requires local release, as intra-articular injection would (1) limit the magnitude and duration of dosing and (2) increase the risk of adverse reactions, including arthrofibrosis and joint stiffness. Therefore, we next explored the effect of TGF-β on MSC chondrogenesis when delivered as a soluble supplement within the medium or when preloaded within the GelMA hydrogel. MSC-seeded GelMA constructs were fabricated as described above and cultured up to 42 days. As compared against TGF-β-free controls, TGF-β was either delivered as a medium supplement (10 ng/mL) with medium changes every 3 days, or added at 2 μg/mL to the GelMA immediately prior to photogelation. Importantly, the TGF-β preloaded constructs were then cultured for 42 days in **TGF-β-free** medium. As seen in **Figure 8**, proteoglycan deposition was increased in both TGF-β groups, regardless of delivery method. While the sGAG content was equivalent between TGF-β groups (but superior to controls) at day 21, the constructs cultured in TGF-β-supplemented medium contained higher sGAG content by day 42 as compared to preloaded constructs (quantification results not shown).

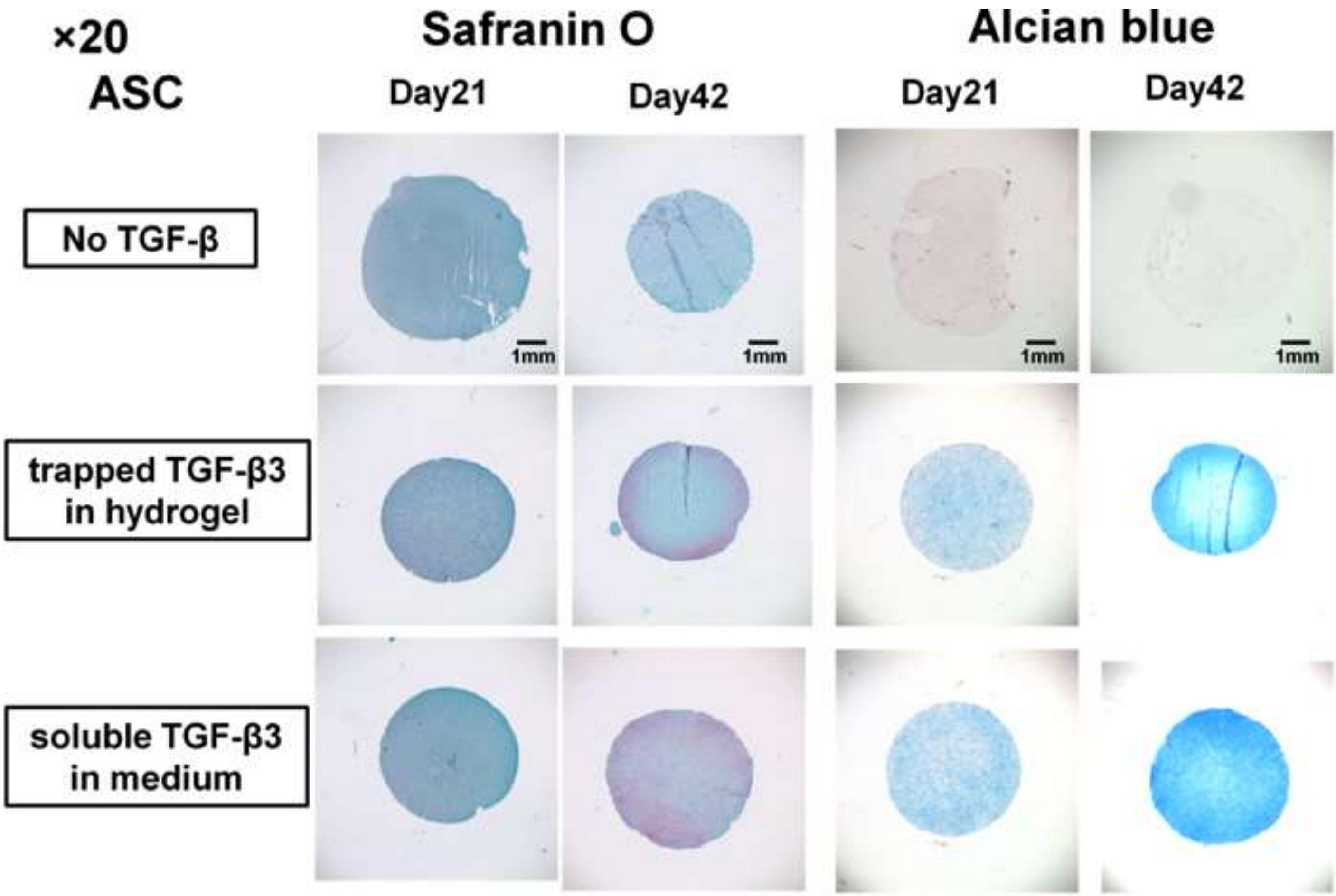
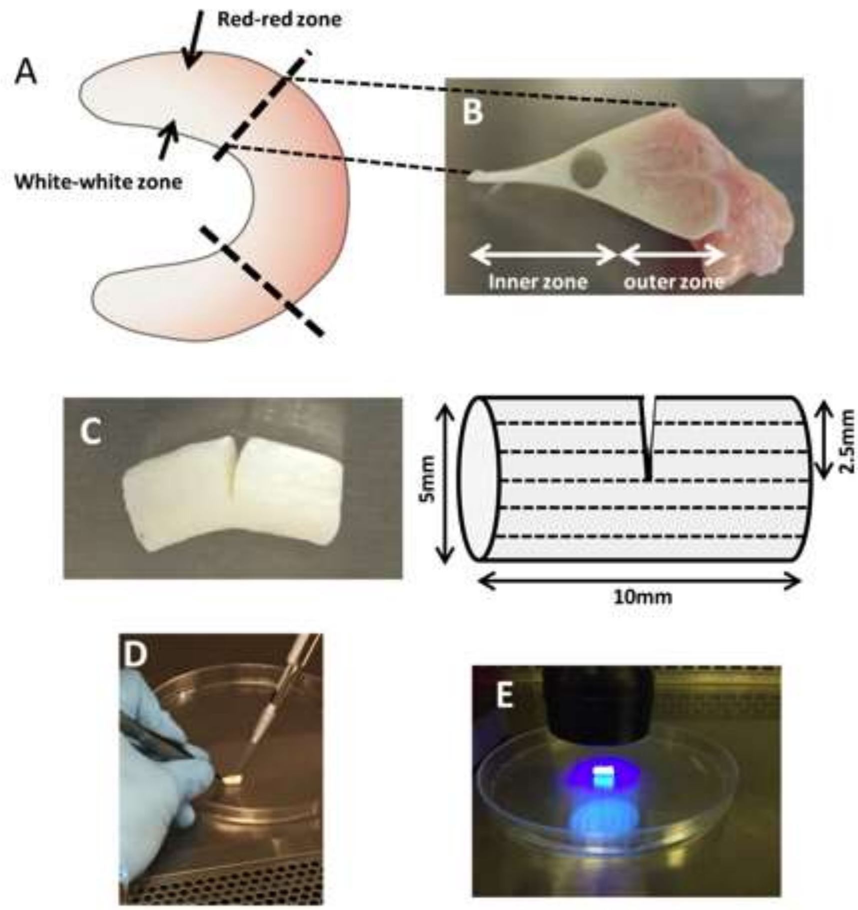


Figure 8. Histology of MSC-seeded constructs. Safranin O (left) and Alcian Blue (right) staining of samples at days 21 and 42.

To investigate the efficacy of these constructs in promoting meniscal repair, we employed our established in vitro explant model, as shown in **Figure 9**. More specifically, a cylinder of inner meniscal tissue measuring 5 mm x 10 mm was obtained by punch biopsy of adult bovine menisci. An incision spanning the radius of the cylinder (2.5 mm) was made to simulate a radial meniscus tear. The lesion was filled with one of six constructs (1) acellular hydrogel alone (Control), (2) acellular hydrogel cultured in TGF-β medium, (3) acellular hydrogel preloaded with TGF-β, (4) MSC-seeded hydrogel, (5) MSC-seeded hydrogel cultured in TGF-β medium, or (6) MSC-seeded hydrogel preloaded with TGF-β. Samples were collected after 4 and 8 weeks and analyzed by histology and immunohistochemistry.



Adopted from Shimomura et al. Tissue Engineering PartA, 2015

Figure 9. Schematic demonstrating in vitro repair model. (A) Using a menisci from adult cows, (B) a cylinder is cored from the inner zone and (C) incised the width of the radius to mimic a radial meniscus tear. (D) The tear is the filled with a photocrosslinkable hydrogel ± MSCs ± TGF-β, which is subsequently gelled by exposure to visible light (E).

As shown in **Figure 10**, the inclusion of MSCs within the hydrogels significantly improved in vitro meniscal tear repair (bottom row of histology), as quantified by the adhesion index. Repair was further enhanced by the inclusion of TGF-β3, regardless of whether delivered as a soluble medium supplement or preloaded within the hydrogels. Given these results, coupled with the putative synergism of TGF-β3 and imECM, as described in Aim 2, we explored the benefit of an MSC-seeded hydrogel preloaded with TGF-β3 and imECM in augmenting healing in a goat model of a radial meniscus tear.

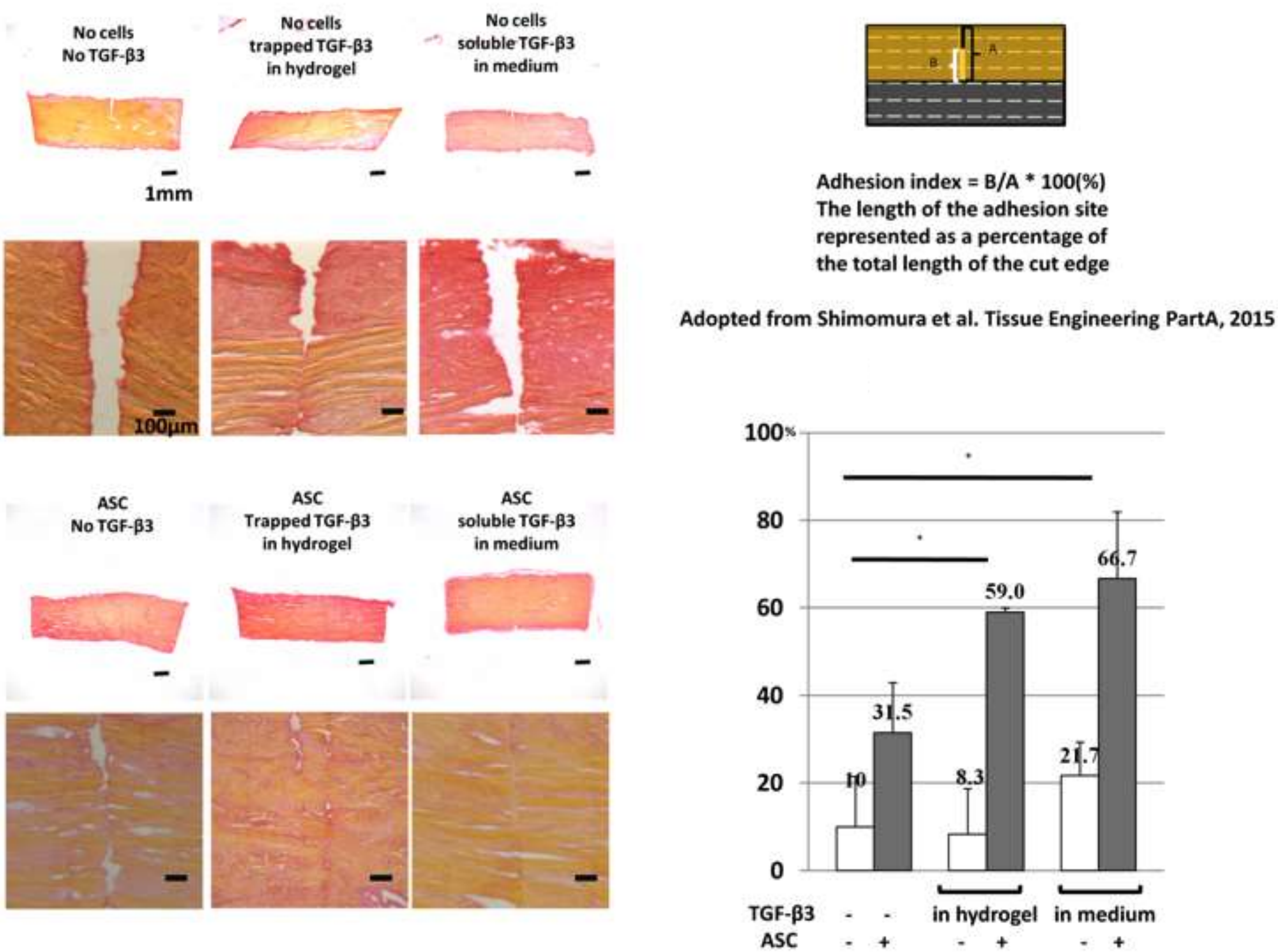


Figure 10. In vitro repair model at 8 weeks. (Left) Histological sections of the defect site at 8 weeks stained with picrosirius red. (Top right) Calculation of adhesion index as a measure of in vitro "healing". (Bottom right) Inclusion of MSCs and TGF-β enhanced in vitro healing. Bars indicate statistically significant difference vs. control ($p < 0.05$)

In Vivo Repair:

We developed a goat model of a radial meniscus tear, with the medial meniscus of the right knee incised 90% of the meniscus width at the junction of the anterior and middle body of the meniscus. With four animals per group, there were 3 conditions: (1) no repair, (2) suture repair alone, or (3) suture repair with augmentation (i.e., 150 µL photocrosslinked GelMA containing $20x10^6$/mL autologous adipose-derived MSCs, 2 µg/mL TGF-β3, and 500 µg/mL imECM). Of note, the MSCs were isolated from the infrapatellar fat pad of each goat, necessitating only one incision for cell isolation, defect creation, and surgical repair. Furthermore, the cell isolation and scaffold fabrication were done intraoperatively, constituting a single-step point-of-care procedure to augment meniscal repair. Goats were euthanized at 6 months and the following analyses were performed (or are ongoing): (1) MRI, (2) macroscopic evaluation of menisci and articular cartilage, (3) histological evaluation of menisci and articular cartilage, (4) mechanical testing of articular cartilage.

As shown in **Figure 11** and the accompanying table, MRI revealed improved healing with suture repair, further enhanced when repairs were augmented with the ASC-hydrogel. A similar result was found when examining the menisci both macroscopically and histologically. As represented in **Figure 12**, thin translucent tissue was frequently seen in the defect site of untreated knees. Conversely, menisci repaired with suture showed improved tissue apposition, but consisted primarily of fibrovascular scar. On the other hand, augmented repairs more consistently contained fibrocartilaginous tissue at the defect site that resembled the adjacent native meniscus.

Figure 11. Representative images of T2-weighted MRI at 6 months. Yellow area indicates hyperdensity consistent with a persistent tear.

	Untreated (n=4)	Suture repair (n=4)	Augmentation (n=4)
No tear	0	0	2
Tear of partial thickness	1	2	2
Tear of full thickness	3	2	0

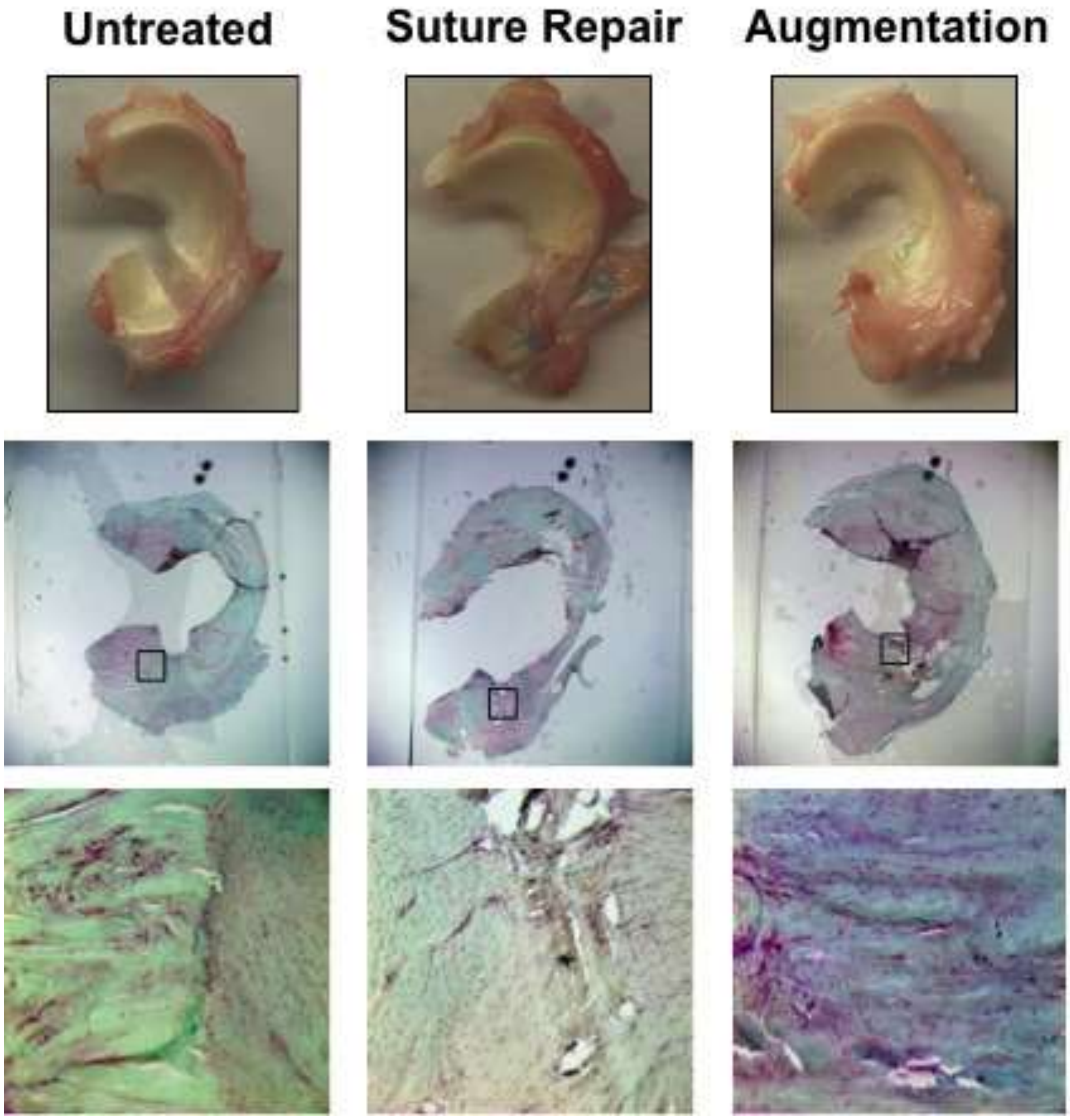


Figure 12. Macroscopic images and histology (Safranin O) of menisci at 6 months. Images in third row are magnifications of tear site, as indicated by black box in second row.

The effect of each intervention on articular cartilage integrity was also examined using India Ink staining and a macroscopic scoring scale developed by Inoue et al. As shown macroscopically, considerable cartilage degeneration was seen on both the medial femoral condyle and medial tibial plateau of the untreated and suture repair groups (**Figure 13**). Conversely, cartilage integrity was better preserved in the augmented groups, as clearly demonstrated by semi-quantitative scoring (bar graph, bottom right). Mechanical testing of the articular cartilage has been performed and is under analysis. Additionally, histological analysis of the articular cartilage is ongoing.

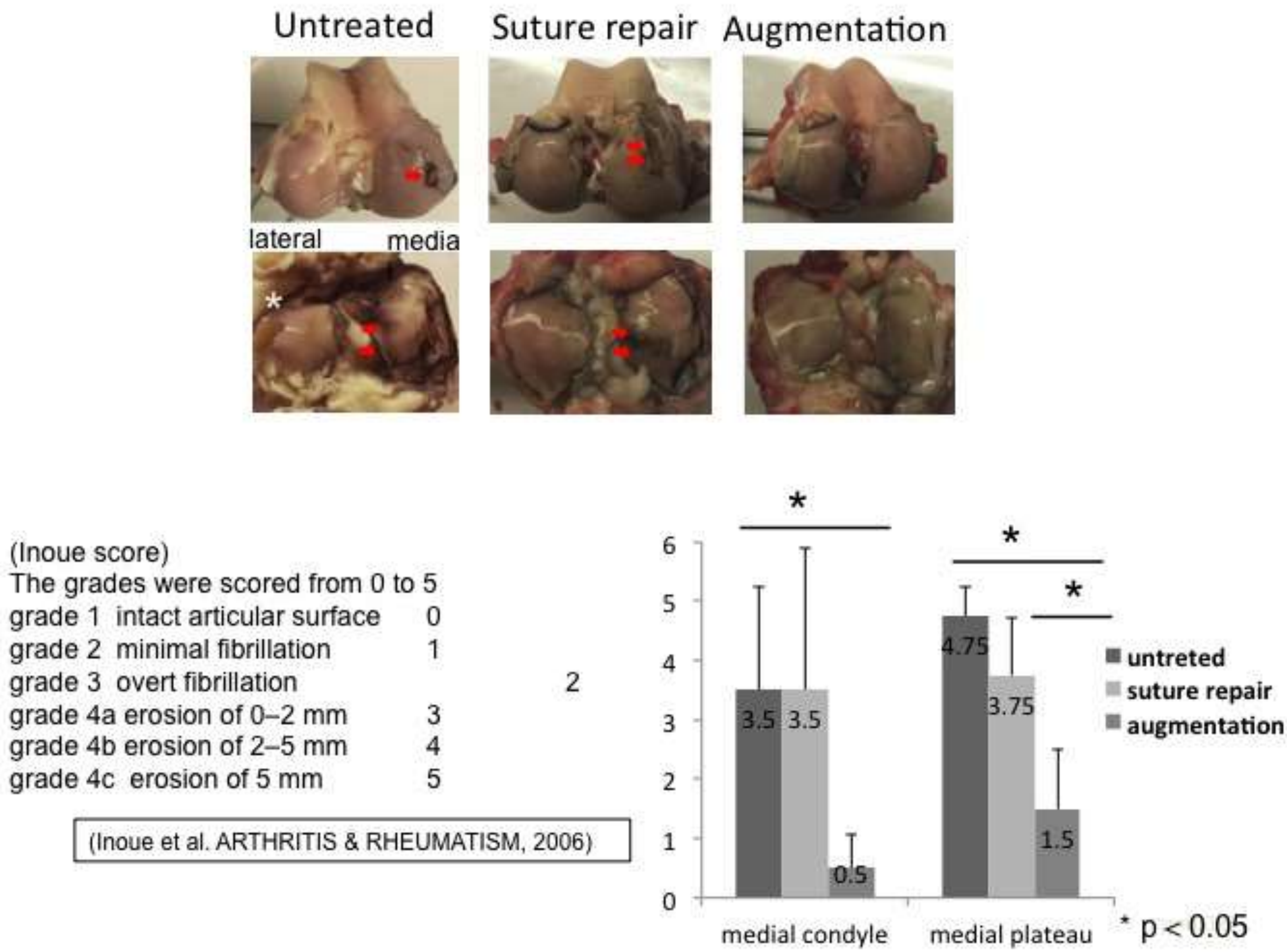


Figure 13. Articular cartilage integrity. Top panels show gross images of femoral condyles (top row) and tibial plateaus (bottom row) stained with India Ink. A semi-quantitative scoring system was adopted from Inoue et al. (bottom left). Results are shown bottom right. * $p < 0.05$.

Aim 3 Discussion:

Through several in vitro studies, we've successfully demonstrated the utility of delivering MSCs and TGF-β encapsulated within a photocrosslinkable hydrogel as a means of promoting 'repair' of a radial meniscus tear. While MSCs and growth factors improve tissue apposition when provided independently, there is a synergistic effect when they are combined. Equally important, we've shown that the delivery of TGF-β within the hydrogel exerts a comparable effect on MSC chondrogenesis and in vitro repair as when TGF-β is delivered as a soluble supplement with each medium change. This has tremendous potential for targeted therapy of focal meniscal lesions.

Building on these in vitro results, we successfully developed a large animal (goat) model of a radial meniscus tear, which has heretofore not been achieved. While several groups have developed caprine/ovine models of meniscal replacement (with either allografts or engineered grafts), the patient population indicated for meniscal replacement is an order of magnitude less than the population that could benefit from meniscal repair. Furthermore, improved healing in the context of meniscal repair would greatly reduce the number of patients

who would ultimately need meniscus replacement. For these reasons, we believe that the development of a large animal model for primary meniscal repair is timely. To that end, we've also shown that a 90% radial tear (if left untreated) does not spontaneously heal and is associated with degeneration of articular cartilage within the same compartment, thereby replicating human pathophysiology.

While the sample size is small (n=4 per group), and the results remain incomplete, preliminary analyses indicate improved meniscus healing and associated chondroprotection in the augmented group. Of note, the adipose-derived ASCs encapsulated within the hydrogel were harvested from the ipsilateral fat pad at the start of the surgery, necessitating only one surgical intervention and performed through a single incision. While this approach provides an autologous source of MSCs (with promising results demonstrated above), isolation of the cells from their surrounding matrix takes at least one hour, significantly increasing operative times. Future studies should consider the use of allogeneic cells or the development of biomaterials capable of promoting endogenous MSC recruitment to the meniscal lesion. Additionally, the GelMA hydrogel itself possesses good compressive properties, but poor tissue adhesivity. Whether hydrogels composed of different polymers would provide a more optimal solution remains an open question. Alternatively, further refinement of a nanofibrous scaffold sheet could provide a mechanism to maintain hydrogel positioning, although it is unknown as to whether the hydrogels utilized in this study migrated from the wound site following initial implantation.

Further discussion of study limitations and future directions will be deferred until completion of study analyses. At present, only histological analysis of articular cartilage remains to be performed, which will be completed in the coming months. Thereafter, we will draft and submit a manuscript by December 2017. Additionally, two manuscripts detailing our in vitro studies are currently under preparation.

What opportunities for training and professional development did the project provide?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Work from the project has been presented at the following conferences:

1. Numpaisal P, Rothrauff BB, Lauro BB, Alexander PG, Debski RE, Musahl V, Tuan RS. "Augmented Repair of Radial Meniscus Tear with Biomimetic Electrospun Scaffold: An In Vitro Mechanical Analysis." 2016 Penn Cartilage Research Symposium, **April 29-30, 2016**. Philadelphia, PA
2. Rothrauff BB, Shimomura K, Gottardi R, Alexander PG, Tuan RS. "Encapsulation of Mesenchymal Stem Cells in Photocrosslinkable Hydrogel Enhanced with Meniscal Extracellular Matrix for Augmented Meniscus Repair." Military Health System Research Symposium, **August 15-18, 2016**. Kissimmee, FL.
3. Sasaki H, Rothrauff B, Alexander PG, Lin H, Fu FH, Tuan RS. (2017) Meniscus repair using hydrogels seeded with stem cells and meniscus extra cellular matrix. Orthopaedic Research Society Annual Meeting 2017. **March 19-22, 2017.** San Diego, CA, USA.
4. Sasaki H, Rothrauff B, Alexander PG, Lin H, Shimomura K, Fu FH, Tuan RS. (2017) In vitro repair of meniscus radial tear using hydrogels seeded with adipose-derived stem cells and TGF-β3. Orthopaedic Research Society Annual Meeting 2017. **March 19-22, 2017.** San Diego, CA, USA.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Results of studies comprising the project have been, and will be, presented at national and international conferences, as outlined above.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The last goat was euthanized in February 2017. Final analyses of mechanical properties and histology are ongoing. Once completed, we will draft and submit a manuscript.

4. IMPACT: This component is used to describe ways in which the work, findings, and specific products of the project have had an impact during this reporting period. Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- the development of the principal discipline(s) of the project;
- other disciplines;
- technology transfer; or
- society beyond science and technology.

Nothing to report

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (*Scientific American style*).

Nothing to report

How the field or discipline is defined is not as important as covering the impact the work has had on knowledge and technique. Make the best distinction possible, for example, by using a "field" or "discipline," if appropriate, that corresponds with a single academic department (i.e., physics rather than nuclear physics).

Nothing to report

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- transfer of results to entities in government or industry;
- instances where the research has led to the initiation of a start-up company; or
- adoption of new practices.

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- improving public knowledge, attitudes, skills, and abilities;
- changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or
- improving social, economic, civic, or environmental conditions.

Nothing to report

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

- Changes in approach and reasons for change.
- Actual or anticipated problems or delays and actions or plans to resolve them.
- Changes that have a significant impact on expenditures.
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

In aim 1, the mechanism of repair failure (i.e., suture breakage) suggested that further changes in scaffold design to enhance material properties and/or suture retention strength would not translate into improved surgical repair strength. Nevertheless, the biomimetic scaffold, when seeded with MSCs, was capable of supporting cell attachment, proliferation, and contact guidance of cell morphology. These findings, coupled

with the promising results of MSC-seeded GelMA hydrogels enhanced with imECM and TGF-β in terms of promoting meniscus-specific neotissue formation, caused us to pursue the following augmentation strategy *in vivo* – (1) an acellular biomimetic scaffold to envelop the tear site, in combination with (2) an autologous MSC-seeded TGF-β/imECM enhanced GelMA hydrogel localized to the tear site. Upon additional pilot testing, we were unable to stably incorporate the electrospun scaffold. This was attributable in part to (1) the small size of the goat knee and (2) limited exposure; namely we tried to preserve the medial retinaculum to reduce the risk of lateral patella luxation. Other groups, when investigating meniscus replacement, have performed medial condylectomy. While this obviously provides great exposure, complications can be catastrophic, necessitating early and immediate euthanasia. Additionally, in keeping with the ultimate goal of developing a minimal invasive procedure to restore meniscal structure and function (thereby preventing further degeneration and the need for meniscal replacement), we tried to minimize surgical exposure. This strategy, while slightly different from the originally approved protocol, does not change the objectives or scope of the project.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Development and testing of the NFSs (Aim 1) and MSC-seeded hydrogel (Aim 2) took longer than 12 months, delaying the start of the *in vivo* experiments (Aim 3). With a 6 month endpoint, Aim 3 will extend beyond the 18-month funding period (December 2016), and additional time will be needed to process the specimens and interpret results. We do not anticipate any challenges in completed the study by the end of the no-cost extension period (December 2017)

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

The delay in beginning the *in vivo* experiments (Aim 3), as described above, caused expenditures for animal housing and care to extend beyond the funding period. However, we anticipate being able complete the project with the approved budget total.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Two small modifications were submitted to the IACUC office, and were subsequently sent to ACURO. These changes do not impact the execution or scope of the project and include:
(1) The use of goat coats post-operatively to reduce the risk of seroma formation at the abdominal site from which adipose tissue will be harvested
(2) Use of banamine and excede (in place of ketoprofen and cefazolin) for post-operative analgesia and anti-biosis. These changes were suggested by the house veterinarian and are currently employed under veterinarian exception.

6. PRODUCTS: List any products resulting from the project during the reporting period. Examples of products include:

- publications, conference papers, and presentations;
- website(s) or other Internet site(s);
- technologies or techniques;
- inventions, patent applications, and/or licenses; and
- other products.

If there is nothing to report under a particular item, state "Nothing to Report."

Nothing to report

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award. There is no restriction on the number. However, agencies are interested in only those publications that most reflect the work under this award in the following categories:

Journal publications. List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Include any peer-reviewed publication in the periodically published proceedings of a scientific society, a conference, or the like. A publication in the proceedings of a one-time conference, not part of a series, should be reported under "Books or other non-periodical, one-time publications."

Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

This project has produced 3 publications to date:

1. Rothrauff BB, Numpaisal P, Lauro BB, Alexander PG, Debski RE, Musahl V, Tuan RS. Augmented Repair of Radial Meniscus Tear with Biomimetic Electrospun Scaffold: An In Vitro Mechanical Analysis. *Journal of Experimental Orthopaedics*. 2016; **3(1): 23**. PMID: 27624439
2. Shimomura K, Rothrauff BB, Tuan RS (2016) Region-specific effect of decellularized meniscus extracellular matrix on mesenchymal stem cell-based meniscus tissue engineering. *American Journal of Sports Medicine*. 2016; **45(3): 604-611.** PMID: 27895039
3. Rothrauff BB, Shimomura K, Gottardi R, Alexander PG, Tuan RS. Anatomical region-dependent enhancement of 3-dimensional chondrogenic differentiation of human mesenchymal stem cells by soluble meniscus extracellular matrix. *Acta Biomaterialia*. 2016; **49: 140-151**. PMID: 27876676

****We anticipate three additional manuscripts to be submitted by the end of 2017***

Books or other non-periodical, one-time publications. Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like.

Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Other publications, conference papers, and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- data or databases;
- biospecimen collections;
- audio or video products;
- software;
- models;
- educational aids or curricula;
- instruments or equipment;
- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Provide the following information on participants:

- what individuals have worked on the project?
- has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?
- what other organizations have been involved as partners?

Nothing to report

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).

- Provide the name and identify the role the person played in the project. Indicate the nearest whole person month (Calendar, Academic, Summer) that the individual worked on the project. Show the most senior role in which the person worked on the project for any significant length of time. For example, if an undergraduate student graduated, entered graduate school, and continued to work on the project, show that person as a graduate student, preferably explaining the change in involvement.

  Describe how this person contributed to the project and with what funding support. If information is unchanged from a previous submission, provide the name only and indicate "no change".

  Example:

Name:	Mary Smith
Project Role:	Graduate Student
Researcher Identifier (e.g., ORCID ID):	1234567
Nearest person month worked:	5
Contribution to Project:	Ms. Smith has performed work in the area of combined error-control and constrained coding
Funding Support:	The XYZ Foundation (Complete only if the funding support is provided from other than this award.)

Name:	Rocky S. Tuan
Project Role:	PI
Research Identifier:	University Employee ID# 124200
Nearest person month worked:	3.75% effort (0.45 Person Months)
Contribution to Project:	Dr. Tuan will have direct responsibility for the overall design and conduct of the study, oversight of data analysis and writing of publications and research reports. Dr. Tuan will supervise the day-to-day research activities of all personnel.
Funding Support:	N/A
Name:	Peter Alexander
Project Role:	Data Analyst Scientist
Research Identifier:	University Employee ID# 124097
Nearest person month worked:	4.25% effort (0.51 Person Months)
Contribution to Project:	Dr. Alexander's responsibilities will include biomaterial scaffold fabrication, cell isolation/propagation/characterization, tissue harvesting/repair/culture, and histological, biochemical, and mechanical analyses, and animal surgeries. He will work under close supervision of Dr. Tuan and will be involved in experimental design, data analysis, and the training of the postdoctoral fellow. He will also be involved in data analysis, and presentation of research findings in manuscripts and at scientific meetings.
Funding Support:	N/A
Name:	Alessandro Pirosa
Project Role:	Postdoctoral Associate
Research Identifier:	University Employee ID# 160892
Nearest person month worked:	20% effort (2.40 Person Months)
Contribution to Project:	Alessandro assists in the execution of the experiments in this project for all the proposed tasks, including biomaterial scaffold fabrication, cell isolation/propagation/characterization, tissue harvesting/repair/culture, and histological, biochemical, and mechanical analyses, and animal surgeries. He has been trained by Dr. Alexander, and will be supervised directly by Dr. Tuan and Dr. Alexander in all of his research activities, including experimental design, assays, and data analyses. He will also be responsible for safety requirement, material acquisition, protocol development, and handle reporting duties according to Department of Defense protocols.
Funding Support:	N/A
Name:	Benjamin Rothrauff
Project Role:	Graduate Student Researcher
Research Identifier:	University Employee ID# 130053
Nearest person month worked:	16.67% effort (2.00 Person Months)
Contribution to Project:	Ben assists in the execution of the experiments in this project for all the proposed tasks, including biomaterial scaffold fabrication, cell isolation/propagation/characterization, tissue harvesting/repair/culture, and histological, biochemical, and mechanical analyses, and animal surgeries. He has been trained by Dr. Alexander, and will be supervised directly by Dr. Tuan and Dr.

	Alexander in all of his research activities, including experimental design, assays, and data analyses. He will also be responsible for safety requirement, material acquisition, protocol development, and handle reporting duties according to Department of Defense protocols.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Rocky Tuan:

None.

Pete Alexander:

The following previously active grant has closed:

None

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission.

Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:

<u>Organization Name:</u>

<u>Location of Organization: (if foreign location list country)</u>

<u>Partner's contribution to the project (identify one or more)</u>

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other.

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

REFERENCES

1. Yang G, Rothrauff BB, Lin H, et al. 2013. Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix. Biomaterials 34: 9295-9306.
2. Shimomura K, Bean AC, Lin H, et al. 2015. In Vitro Repair of Meniscal Radial Tear Using Aligned Electrospun Nanofibrous Scaffold. Tissue Eng Part A 21: 2066-2075.